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A virological investigation into declining woylie populations

Running head: Virological investigation into woylie populations

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Abstract

The woylie (*Bettongia penicillata ogilbyi*) is a critically endangered small Australian marsupial that is in a state of accelerated population decline for reasons that are currently unknown. The aim of the present study was

23 to elucidate the involvement of several viral pathogens through strategic serological testing of several wild
24 woylie populations. Testing for antibodies against the Wallal and Warrego serogroup of orbiviruses,
25 Macropod herpesvirus 1 and Encephalomyocarditis virus in woylie sera was undertaken through virus
26 neutralisation tests. Moreover, testing for antibodies against the the alphaviruses Ross River virus and Barmah
27 Forest virus and the flaviviruses Kunjin virus and Murray Valley encephalitis virus was undertaken through
28 virus neutralisation tests and ELISA mainly because of the interest in the epidemiology of these important
29 zoonoses as it was considered unlikely to be the cause of the decline. Between 15 and 86 samples were tested
30 for each of the four sites in south-western Australia (Balban, Keninup, Warrup and Karakamia). Results
31 indicated no exposure to any of the viral pathogens investigated, indicating that all populations are currently
32 naïve and may be at risk if these pathogens were to be introduced.
33
34

35 # Introduction

36 Diseases can regulate the demography of wildlife species and represent, by themselves or in association with
37 other factors, a potential cause of decline (Caughley and Gunn 1996; Daszak and Cunningham 1999; Daszak *et*
38 *al.* 2000; Aguirre *et al.* 2002). Emerging infectious diseases in wildlife are reported world wide and include a
39 variety of pathogens such as viruses, parasites and protozoa (Daszak *et al.* 2000; Daszak *et al.* 2001). In
40 Australia various native species are threatened by several diseases (Bunn and Woods 2005; Kirkland 2005;
41 Skerratt 2005; Spratt 2005; Symonds 2005) with most of these having viral aetiology, including orbiviruses and
42 herpesviruses (Kirkland 2005).
43

44 The woylie (*Bettongia penicillata ogilbyi*) is a critically endangered small Australian marsupial that has recently
45 undergone a dramatic decline with all high density populations being affected. Naturally occurring
46 populations had been limited to three sites in Western Australia. Of these, during the course of this study, one
47 went locally extinct, and the other two have declined by more than 90% (Wayne *et al.* 2013a).
48
49 Detailed demographic data were available in the Upper Warren region (Fig. 1), enabling the demonstration
50 that the decline had peculiar spatial and temporal characteristics. The decline progressed northerly at an
51 average rate of 4 km per year and little difference was found between the yearly rates of decline over time
52 among forest blocks in the Perup Nature Reserve (Fig. 2), which includes the eastern sites of the Upper
53 Warren region (Wayne *et al.* 2013b). Predecline density was always high (> 1 woylie ha^{-1}) (Wayne *et al.*
54 2013b). The decline progressed over a period of 3-5 years in each forest block and when the density reached a
55 low level, it appears to have stabilised (Wayne *et al.* 2013b).
56
57 Given the characteristics and demographics of the woylie decline, Wayne *et al.* (2013b) hypothesised that,
58 while predation by introduced predators played an important role, a disease may be a concomitant, if not
59 primary, cause of the decline. Therefore, a disease investigation was initiated as part of the woylie
60 conservation and research project.
61
62 The determination of the presence of a pathogen in a population and its prevalence are the initial steps in a
63 disease investigation (Artois *et al.* 2001). These allow the prioritisation of future research aimed to quantify
64 the effects of specific pathogens at a population level. An initial qualitative assessment of the hazard posed by
65 potential diseases was carried out to aid the woylie disease investigation (Pacioni 2010) and, for the reasons
66 briefly outlined below, it was deemed critical to determine whether detectable immune responses to

67 macropodid herpesviruses, encephalomyocarditis virus (EMCV) or Orbiviruses (Wallal and Warrego
68 serogroups) were present.
69
70 Most macropodid herpesviruses (MaHV-1 and MaHV-2 and the recently discovered MaHV-4; Johnson and
71 Whalley 1987; Johnson and Whalley 1990; Vaz *et al.* 2013) belong to the subfamily *Alphaherpesvirinae*.
72 Members of this subfamily generally spread rapidly and establish a latent infection in spinal or cranial nerve
73 ganglia (Quinn *et al.* 2002). As with other herpesviruses, MaHV can reactivate as a result of immune
74 suppression or other stimuli (Guliani *et al.* 1999). Herpesviruses are usually species-specific and highly
75 adapted to their hosts and the hypothesis of co-evolution of these viruses with their hosts is well accepted
76 (McGeoch *et al.* 1995; Mahony *et al.* 1999). However, the phylogenetic relationship of MaHV-1 and 2 with
77 other viruses of the subfamily would appear to contradict the co-evolution theory (Mahony *et al.* 1999). Yet in
78 spite of this, only macropod species have been found to be susceptible to infection with MaHV. For example,
79 inoculation of MaHV-1 failed to establish a systemic infection in the common brushtail possum (*Trichosurus*
80 *vulpecula*) (Zheng *et al.* 2004).
81
82 Recently, a new gammaherpesvirus, *Macropodid herpesvirus 3* (MaHV-3) was described after being isolated
83 from eastern grey kangaroos (*Macropus giganteus*) (Smith *et al.* 2008; Wilcox *et al.* 2011). This virus was not
84 available for use in this study and therefore it could not be included in the serological evaluation of the woylie
85 sera.
86
87 Macropodoid herpesvirus is present in the regions where woylie populations occur and has resulted in sudden
88 death in captive populations of grey dorcopsis wallabies (*Dorcopsis muelleri luctuosa*), quokkas (*Setonix*
89 *brachyurus*), western grey kangaroos (*Macropus fuliginosus*) and woylies (Dickson *et al.* 1980; Callinan and
90 Kefford 1981; Wilks *et al.* 1981). However, the widespread distribution of antibody titres in marsupials

91 suggests that this virus has evolved with marsupial species and may be endemic in wild populations (Webber
92 and Whalley 1978). High antibody levels in captive animals may reflect a higher level of virus transmission due
93 to crowding, increased contact rates with infected animals, or increased stress leading to expression of latent
94 virus (Webber and Whalley 1978).
95
96 Based on the identification of characteristic inclusion bodies, the nature of the pathology lesions and growth
97 of herpesvirus in cell cultures, Dickson *et al.* (1980) reported an outbreak of MaHV infection that resulted in
98 the sudden death of eight captive woylies, among other species, over a period of a week. This report indicates
99 that woylies are highly susceptible to MaHV infection. Clinical signs, such as ulcerations or vesicles, may be
100 rare and difficult to detect in field investigations and with opportunistic surveillance as conducted with wild
101 woylie populations, it could be difficult to detect the presence of MaHV disease in a population. In this
102 scenario, an affected population could undergo a steep decline, with increased mortality after introduction of
103 the virus, similar to what the available monitoring data suggest.
104
105 The encephalomyocarditis virus (EMCV) is shed in the faeces and urine of rodents that are the natural hosts.
106 The infection develops in a few days and the virus can replicate in a broad spectrum of hosts including
107 primates, pigs, rodents and marsupials (Reddacliff *et al.* 1997; McLelland *et al.* 2001; Quinn *et al.* 2002).
108 Encephalomyocarditis virus is a known pathogen in rural areas in Australia and it can occur when rodents
109 build up to plague proportions around piggeries. The disease in pigs is acute with sudden death or acute
110 neurological signs. EMCV was isolated from a variety of Australian native fauna, including macropods
111 (*Macropus rufogriseus*, *M. rufus* and *Dendrolagus goodfellowi*), causing sudden death as the only sign
112 (Reddacliff *et al.* 1997). More recently, a case of sudden death caused by EMCV was reported in a Lumholtz's
113 Tree Kangaroo (*Dendrolagus lumholtzi*) (David Blyde, pers. comm.), as well as an infection in a quokka
114 (McLelland *et al.* 2001).

Viruses in the genus *Orbivirus* are generally transmitted by arthropods, particularly *Culicoides* species. Infections, especially by the Wallal and Warrego serogroups, have been reported in several species of macropods that are closely related to woylies, with serious clinical consequences (Daszak *et al.* 2000). Therefore, woylies may be potentially susceptible to these viral pathogens. At its extreme, a severe clinical form of orbivirus infection, similar to that described for other kangaroos (e.g. western grey kangaroos), could have critical consequences in woylie populations. A number of orbivirus epidemics were reported in wild populations of macropods in Australia between 1969 and 1996, including outbreaks in the South West region of Western Australia (Hooper 1999; Hooper *et al.* 1999). The virus was isolated from eye and brain tissues collected from diseased kangaroos near Albany, Esperance and Perth (Hooper *et al.* 1999). Hooper et al. (1999) also reported a high seroprevalence of antibodies for these viruses in wild kangaroos and wallabies throughout the region. Consequently, it can be concluded that these viruses are well established in the region.

Lastly, we also tested woylie sera for the presence of antibodies against flaviviruses and alphaviruses (Ross River virus, RRV, and Barmah Forest virus, BFV). The viruses RRV and BFV are regularly isolated from mosquitoes collected in the south-west of Western Australia by the Arbovirus Surveillance and Research Laboratory at the University of Western Australia, and the seroprevalence in marsupials can be high (Lindsay 1995). The flaviviruses Edge Hill virus, Kokobera virus and Stratford virus have also been isolated from mosquitoes in the south-west of Western Australia, however little is known about the prevalence of antibodies against these pathogens in native fauna in the southwest of Western Australia (Jasinska *et al.* 1997; Johansen *et al.* 2005a). Macropods are known to have been infected with specific flaviviruses and alphaviruses but have not shown any evidence of disease, and sero-epidemiological studies suggest they could be possible virus reservoirs (Aldred *et al.* 1990; Harley *et al.* 2001; Russell 2002; Johansen *et al.* 2005b).

Knowing whether this species of macropod is contributing to maintain the virus in the habitat would also be of great interest in terms of human health as some of the infections caused by these viruses are important zoonoses. Furthermore, a positive serological result could give an indication of the exposure of this species to biological vectors such as mosquitoes. For these reasons we also included this group of viruses in the serological screening conducted in this study.

Materials and Methods

Woylies were trapped using a standard live cage-trapping technique in the Upper Warren region and Karakamia sanctuary (Fig. 1) between March 2006 and November 2008. Blood samples were collected from the lateral tail vein via a 23- or 25-gauge needle in a 2.5 or 3 ml syringe and then transferred into plain tubes. After blood clots had formed, samples were chilled in an insulated foam container with ice in the field. Upon return from the field, blood samples were centrifuged as soon as possible after formation of blood clots (but never later than 8 h after collection), and sera separated and frozen at -20°C until tested.

The selection of samples to be tested for specific tests required careful determination given the small amount of serum obtained from sampled animals and the limited number of samples available, especially from low density forest blocks. Under the hypothesis that the tested virus was the cause of the decline, two main criteria were used to select the samples: (1). The pathogen should be present in all forest blocks because it was theorised that declines within each block were not independent (Wayne *et al.* 2013b); and (2) Seropositive individuals were more likely to be detected in the population after the decline because survivors would be likely to have immunity (Thompson *et al.* 1992; Roelke-Parker *et al.* 1996; Tompkins *et al.* 2002; Härkönen *et al.* 2006; Thrusfield 2007).

161

162 Consequently, only postdecline samples from Balban forest block (Fig. 2) were tested for EMCV and the two
163 *Orbivirus* serogroups. Woylies from a broader geographical area were tested for MaHV including a control
164 population (Karakamia) and 15 samples were predecline (Keninup forest block). The population at Karakamia
165 was considered a control population because this is a high density population that has not declined. It would
166 be expected, therefore, that this population would not show evidence of exposure to the virus. When
167 available, multiple samples collected at different times from the same individual were also tested (Table 1).
168 The maximum possible prevalence (i.e. power analysis) with 90 and 95% confidence was calculated following
169 the methods of Cannon and Roe (1982), using Episcope 2.0 (Thrusfield *et al.* 2001) with an estimated
170 population size of 1,000 (Groom 2010).

171

172 All sera were heat inactivated at 56°C for 30 min before to testing.

173

174 For MaHV, virus neutralisation tests (VNTs) were carried out as follows: 10 µL of sera were diluted in 20 µL of
175 phosphate buffer solution (PBS) and mixed with 10 µL MaHV-1 control virus containing 100 $TCID_{50}$ of the
176 respective MaHV-1 and incubated at 37°C for one hour. The virus/serum mixture was then added, in 24 well
177 plates (Nunc, Roskilde, Denmark), to the monolayer of *Potorous tridactylus* kidney cells previously washed
178 from the media with sterile PBS. After the addition of 1 mL of maintenance media, the cell culture plates were
179 incubated at 37°C in an atmosphere containing 5% CO_2. A 'cells-only' well (i.e. a well with no virus and no
180 serum) and virus control was included in each plate. Plates were observed daily for 7 days for presence of
181 cytopathic effect (CPE) in the monolayer. It should be noted that antibodies against MaHV-2 and MaHV-4
182 would also neutralise also MaHV-1, although with different sensitivity (Vaz *et al.* 2013).

183

184 The VNTs for EMCV and the two *Orbivirus* serogroups were carried out in 96 well tissue culture plates (Cooke
185 Engineering Co., Alexandria, VA). Dilution series (from 1:4 to 1:32) of sample sera were mixed with a constant
186 virus concentration of 100 $TCID_{50}$/25 uL and baby hamster kidney (EMCV) or BSR (*Orbivirus*) cells suspended
187 in Dulbeccos' Modified eagle Medium (DMEM; EMCV) or a 1:1 mix of DMEM and Basal Medium Eagle
188 (*Orbivirus*), and incubated at 37°C in an atmosphere containing 5% CO_2. A control plate was used for each
189 test. This included a positive and negative control, a dilution series of the positive control, cell control wells
190 with no added virus or serum, a virus titration, and a working strength titration. Additionally, sample sera
191 were diluted 1:4 with media and an aliquot was added to a well with no virus to check for toxic effects on the
192 cell cultures. Within 24-48 hours of incubation, cell cultures were checked for proper attachment to the
193 bottom of the well and at the fifth day for CPE.
194
195 Woylie sera were screened for the presence of antibodies to RRV and BFV by VNT as described by Johansen et
196 al. (2005b) with the exception that Vero cells were used instead of BHK cells. Briefly, serially diluted sera were
197 incubated with 50-100 $TCID_{50}$s of virus diluted in M199 (supplemented with HEPES, antibiotics and L-
198 glutamine) containing 2% fetal bovine serum (FBS) at 37^{o}C in a 5% CO_2 atmosphere. After one hour
199 approximately 1.6 x 10^4 Vero cells in M199 containing 10% FBS were added to each well of the 96 well tissue
200 culture plate and the plates were incubated for five days at 37^{o}C in a 5% CO_2 atmosphere. Each well was
201 examined microscopically for CPE and neutralisation titres were expressed as the reciprocal of the highest
202 serum dilution where CPE did not occur. Samples with neutralisation titres of 40 or more were considered
203 positive. Virus control assays were performed each time the neutralisation assay was conducted and the assay
204 was repeated if the infectious titre of virus used was below or above 50-100 $TCID_{50}$s. Each test serum was also
205 added to wells containing Vero cells without virus as serum controls, and kangaroo and rabbit sera containing
206 antibodies to RRV and BFV, respectively, were used as positive control sera.
207

208 The presence of flavivirus antibodies in woylie sera were investigated using the flavivirus specific mouse
209 monoclonal antibody 3H6 in a flavivirus epitope blocking ELISA (Hall *et al.* 1995). Optimal concentrations of
210 cell lysate antigen, 3H6 and horse-radish peroxidise (HRPO)-conjugated goat anti-mouse antibody was initially
211 determined using a checkerboard assay. Cell lysate antigen (MVEV) in 0.05 M carbonate/bicarbonate coating
212 buffer pH 9.6 was added to U-bottom flexible plates overnight at 4^{o}C, leaving two wells containing buffer
213 alone as background inhibition control wells. After removing excess antigen and washing twice with PBS
214 containing 0.05% Tween 20 (ELISA wash buffer), 50 µl of woylie test sera and positive and negative control
215 chicken sera diluted 1/10 in ELISA blocking buffer (0.05 M Tris, 1 mM EDTA, 0.15 M NaCl, 0.05% Tween 20,
216 0.2% high nitrogen casein, pH 8.0) were added to duplicate wells on each plate. Plates were incubated for two
217 hours at room temperature, before 50µl of 3H6 diluted in ELISA blocking buffer was added to each well. Plates
218 were incubated for one hour at room temperature before plates were washed four times to remove excess
219 serum and antibody. Fifty microlitres of HRPO-conjugated goat anti-mouse antibody diluted in ELISA blocking
220 buffer was added to each well for one hour at room temperature before being washed six times. Enzyme
221 activity was visualised by the addition of 100 µl of 2,2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS)
222 substrate buffer (Hall et al. 1995) for one hour at room temperature and results were obtained quantitatively
223 by measuring the optical density at a dual wavelength of 415 and 490 nm using a BioRad ELISA plate reader.
224 The percentage inhibition of 3H6 was calculated for each serum. Samples with a percentage inhibition of 30%
225 or greater were considered positive.
226

227 Results

228 No samples neutralised MaHV-1. Complete degradation of the monolayer was apparent within 5-7 days. No
229 CPE was observed in any cells-only wells.

Similarly, no virus neutralisation was observed in any of the sera samples tested for the presence of antibodies to EMCV or Warrego and Wallal serogroups. Initially two samples tested positive for antibodies to the Wallal serogroup virus to serum ratios of 1:4 and 1:8. However, the virus titre in the virus control was below the target titre so the test was considered invalid. When the test was repeated the samples were both positive at a serum dilution of 1:4, however the challenge virus titre was still just below the target titre. Unfortunately, the lack of sera prevented further repeats of the Wallal serogroup test. At this titre these samples were considered to be negative (Reddacliff *et al.* 1999).

No samples were positive for antibodies to flaviviruses in the epitope blocking ELISA. Similarly, no virus-specific antibodies were detected in the RRV and BFV VNT assays.

Discussion

Overall, there was no serological evidence of any of the tested viruses affecting the populations. However, due to the limited sample size, it was not possible to exclude infection from these viruses but only to exclude a seroprevalence above the maximum possible prevalence (Table 1).

There is direct evidence that woylies are susceptible to MaHV, as infection in a captive colony of woylies housed at the Perth Zoo resulted in a case fatality rate of 100% in one week (Dickson *et al.* 1980). However, there is no information available about possible morbidity and fatality rates caused by MaHV, or the other viruses tested in this study, in wild woylie populations to judge whether disease caused by one of these viruses may be responsible for the declines and result in post-decline prevalences lower than the maximum

252 possible prevalence (3.4-7.5%, Table 1). Additionally, it could be expected that, after an epidemic with a very
253 high fatality rate, as it would be expected with MaHV, only a very small fraction, if any, of the population
254 would have detectable antibody levels, despite the significant regulation that the disease would impose on
255 the population demography. In such a scenario, where susceptible animals were exposed to a highly fatal
256 disease, all, or almost all, infected animals would die and therefore it would not be expected that the disease
257 could be detected (serologically) unless sick animals were trapped prior to death. A similar situation was
258 modelled in fox (*Vulpes vulpes*) populations where, assuming that a viral respiratory infection causes the
259 death of 50% of infected animals, a prevalence of 0.18% would be sufficient to regulate the demography of
260 the populations (Anderson 1995).
261
262 The results of this virological investigation contributed to the baseline data needed to improve the
263 understanding of the population dynamics of woylies and to better assess risks for management of, and
264 translocation among these populations, as well as the establishment of new populations. Regardless of
265 whether these viruses are present in the populations at a lower prevalence than the maximum detectable
266 level from this study, or whether they are completely absent, a substantial proportion of individuals has not
267 been exposed to these viruses and therefore these populations may be particularly susceptible.
268 Anthropogenic introduction of these pathogens in woylie populations must be avoided and preventive
269 measures adopted. While current hygiene protocols adopted by Department of Parks and Wildlife (formerly
270 Environment and Conservation) are extensive (Chapman *et al.* 2011) and transmission of these viruses is
271 unlikely through movements between sites by people and equipment (as long as the protocols are followed
272 meticulously), management protocols to prevent the spread of these diseases when dealing with live animals
273 are established on a case by case basis (Chapman *et al.* 2011). These procedures should include quarantine
274 and routine health screening, and if unhealthy animals are encountered, they should be retained at a

quarantine facility for clinical examination to determine the causes. These recommendations have been standard practice as part of monitoring and research activities in the Upper Warren since 2005 (DEC 2008).

Given the risks, quarantine and viral serological screening is recommended before wild rehabilitated woylies or individuals sourced through a captive breeding program are released back into the wild, if these woylies were housed in direct contact or in proximity to other macropods. Clinical, latent and reactivation infections with MaHV have been widely demonstrated in other species of macropods, especially in captive populations (Webber and Whalley 1978; Finnie 1980; Kerr *et al.* 1981; Guliani *et al.* 1999), and it could have devastating consequences if introduced into woylie populations. When infection with EMCV is potentially possible (for example, captive groups with known presence of rodents in enclosures), the establishment of quarantine periods (associated with good rodent control in the quarantine facility) before releasing woylies into the wild is recommended. This preventive measure can be considered sufficient to stop the introduction of this pathogen into the target population, since, except in rodents, infection with EMCV generally causes sudden death shortly after infection (Reddacliff *et al.* 1997; McLelland *et al.* 2001; Fowler and Miller 2003; Jackson 2003). It is more difficult to put in place a control protocol for *Orbivirus* infections, since these viruses are transmitted by arthropods, making the exposure to these viruses a possible eventuality, especially in areas were Wallal and Warrego viruses are known to occur (e.g. south west Western Australia, Hooper 1999; Hooper *et al.* 1999). However, infection with Wallal and Warrego viruses results in clinical signs in macropods (Fowler and Miller 2003; Jackson 2003), and it would be expected that these signs would be evident during the quarantine period.

Every reasonable effort should also be made to prevent the possibility of wild animals that may be positive for any of the investigated viruses (EMCV, Wallal or Warrego serogroups or MaHV) being translocated to different sites, either elsewhere in the wild or to captive populations. While it may not be practical to screen

and test candidate animals for movement beforehand, prior screening of the source population can provide an indication of the likelihood and risks of individuals within the population being infected—as demonstrated by this study for the woylie populations in the Upper Warren region and Karakamia.

The absence of flavivirus antibodies in woylies is perhaps not surprising as isolations of flaviviruses (including Edge Hill virus, Kokobera virus and the closely related Stratford virus) from mosquitoes collected in the southwest of Western Australia is relatively rare and of unknown epidemiological significance. Serosurveys for flavivirus antibodies in marsupials in this region have not previously been conducted. The only BFV serosurvey in the southwest of Western Australia also suggested BFV infection was rare in the few small native animals tested (Johansen *et al.* 2005b). However, high prevalence of BFV antibodies in kangaroos were detected after an outbreak of BFV in 2000/01 and 2001/02. A previous serological survey of antibodies to RRV showed that this virus was common in western grey kangaroos, however few other marsupials were tested. A single western quoll (or chuditch, *Dasyurus geoffroii*) and four quokkas had antibodies to RRV (Lindsay 1995), suggesting that these species may occasionally become infected with this virus, and that the mosquito vectors of RRV occasionally take blood meals from smaller native animals.

In conclusion, this study provides important baseline information that will aid future population health screenings and inform the necessary precautions that need to be taken during the management of existing populations, future translocations or the establishment of captive populations. The study has also improved our understanding of the likelihood of whether these viruses may have been involved in the recent woylie declines. Further studies will be required to confirm complete naivety of woylie populations to the viruses tested but the data presented here are an important foundation on which to build.

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Figure legends

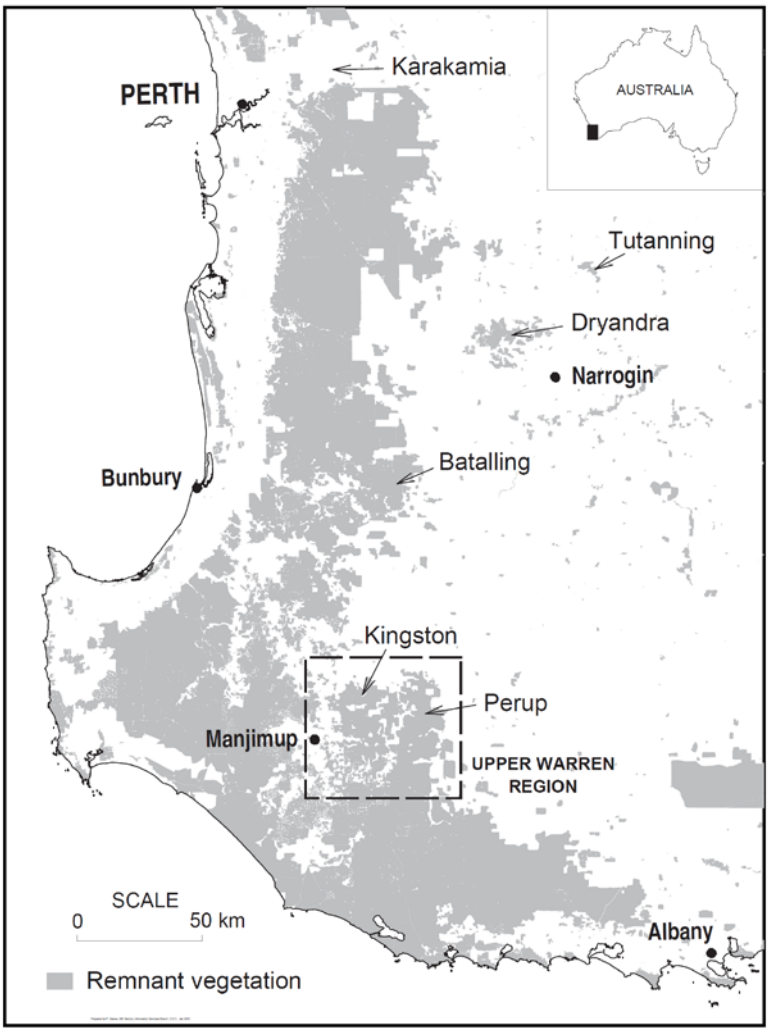
Fig. 1. Location of large woylie populations in Western Australia. Natural occurrring populations: Kingston, Perup, Tutanning and Dryandra. Translocated populations: Karakamia and Batalling.

Fig. 2. Location of the two distinct woylie populations (Pacioni *et al.* 2011) (upper-case letters) and monitored forest blocks (lower case letters) in the Upper Warren region.

Table 1. Summary of samples tested and maximum possible prevalence.

Locations	Balban				Keninup				Warrup	Karakamia			
Virus	EMCV	WAR	WAL	MaHV	MaHV	Flavivirus	RRV	BFV	MaHV	MaHV	Flavivirus	RRV	BFV
Pre-decline	0	0	0	0	15	0	0	0	0	NA	NA	NA	NA
Post-decline	66	44	38	50	48	45	45	44	49	NA	NA	NA	NA
Total individual tested	66	44	38	50	63	45	45	44	49	38	28	28	28
Total individual with multiple samples	16	6	11	11	9	0	0	0	2	0	0	0	0
Total samples	86	51	51	65	72	45	45	44	51	38	28	28	28
Pr 90%Conf	3.40%	5.00%	5.80%	4.40%	3.50%	4.90%	4.90%	5.00%	4.50%	5.80%	7.8%	7.8%	7.8%
Pr 95%Conf	4.30%	6.50%	7.50%	5.70%	4.50%	6.30%	6.30%	6.50%	5.80%	7.40%	10.1%	10.1%	10.1%

EMCV: Encephalomyocarditis virus. WAR: Warrego serogroup. WAL: Wallal serogroups. MaHV: Macropod herpesvirus. RRV; Ross River Virus. BFV: Barmah Forrest Virus. Pr 90%-95%Conf: maximum possible prevalence at 90% and 95% confidence, based on total number of individuals tested and assuming a population size of 1000 animals. Sample sizes reflect availability of sera (i.e. the quantity of sera available was not sufficient to carry out tests for all viruses in all locations).



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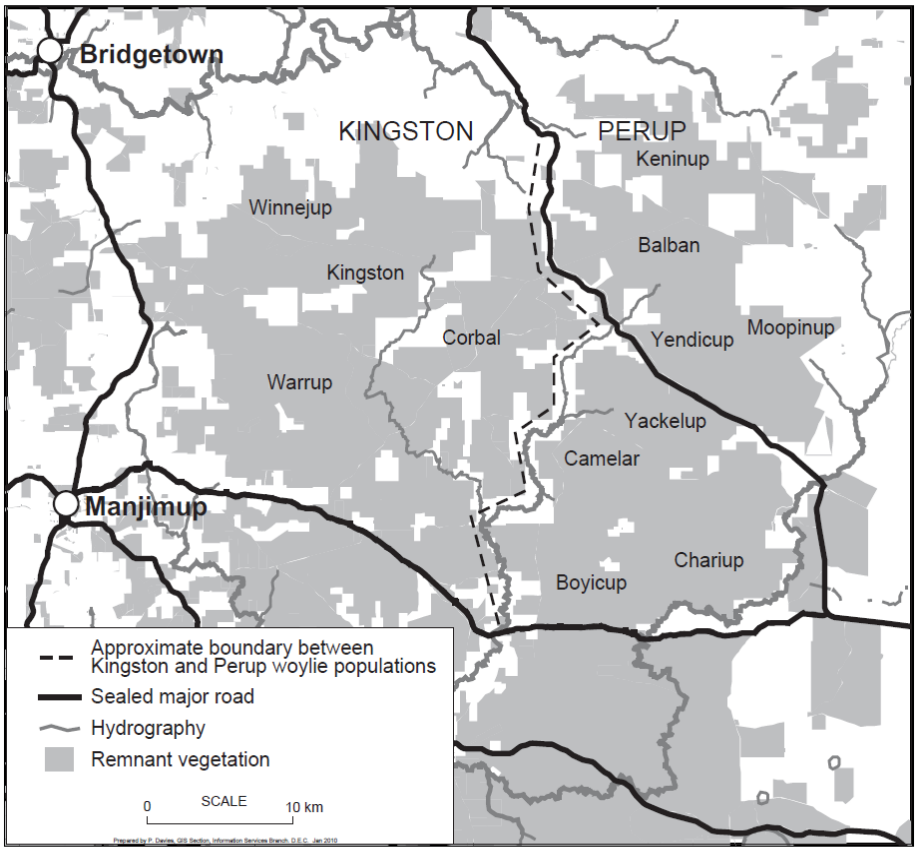

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